Longitudinal monitoring of maternal care and maternal neglect

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Parental care is required to ensure the health and survival of offspring. Caretaking in mammals is challenging as infants are largely helpless and require near-continual oversight over prolonged periods. Mouse pups in particular cannot thermoregulate, and will succumb to hypothermia unless kept warm by an adult caretaker or an insulating nest. Parents must balance caretaking behaviors with other activities important for survival such as regulating their own temperature and foraging for food or water, which necessarily involve periods of pup neglect. To understand in high detail the consequential decision-making involved in care of infant pups, we built a new system for long-term 24/7 continuous monitoring of mouse homecage behaviors, including thermal imaging, video recording from above, side and directly under the nest, with environmental controls to standardize housing conditions across animals. We monitored single dams and their offspring, before, during and after parturition, from initial mating over four consecutive litters. We found that about half of the animals had high litter survival rates ('high-pup-survival' dams) but the other half had little to no pups survive ('low-pup-survival' dams). The relative amount of litter survival did not change or improve across litters in absence of intervention: Low-pup-survival dams continued to neglect pups and nest-building across litters, losing nearly all offspring within a day after birth. We could predict which animals would become low-pup-survival dams by inspecting the nests they built a few days before parturition. Low-pup-survival dams minimally engaged with nest building, and after birth, generally avoided pups and the nest. Nest quality deteriorated due to movement of the dam in and out of the nest, exacerbating temperature loss and mortality of the litter. In contrast, high-pup-survival dams spent a considerable amount of time rebuilding and adjusting the nest over the first postnatal day and beyond. Remarkably, if we co-housed low-pup-survival dams with a high-pup-survival dam and her

litter, when the low-pup-survival dam was bred again and singly-housed, litter survival rates thereafter were consistently much higher along with increased attention toward pups and nest. Thus, even under well-controlled and ideal lab housing conditions, mouse maternal care can be remarkably variable, with continued nest rebuilding as the most important factor in ensuring high litter survival rates. Furthermore, despite some animals perseverating in maternal neglect and losing their pups, caretaking and nest-building can improve after interactions with experienced and successful parents.

Main Text

Parental behavior is essential for the well-being of offspring and the survival of the species (Rilling and Young, 2014). Adult animals can become highly sensitive to the needs of their infants to adequately care for them, as infants require intense continuous protection for days to years depending on species, and single parenting is especially challenging. Mouse pups in particular rely on their mother for sustenance, thermoregulation, and protection, supported by successful nest-building (Lynch and Possidente, 1978). However, pup mortality can result if dams are neglectful or provide inadequate care during the postnatal period. Previous studies of rodents and other species report a remarkably high degree of infant mortality, both outdoors in native habitats and animals housed in laboratory conditions (Brajon et al., 2021). We aimed to determine when and how pup mortality occurs, if it is due to parental neglect or other factors, and if mouse mothers might learn from certain experiences to improve maternal care over subsequent litters.

We designed a high-resolution system to monitor parental care and family life in mice with multiple cameras, recording from homecages continuously 24 hours/day for weeks to months

across consecutive litters (**Fig. 1A**). Individual cages were contained within custom-designed environmental control boxes, containing at least two camera mounts to record the nest from the side, underneath, and overhead views, including infrared cameras to record in darkness and a thermal imaging camera to monitor temperature. Video was combined with an inexpensive, open-source system to control LED light-strips to provide a naturalistic light/dark cycle, together with temperature and humidity sensors connected to an Arduino circuit board that controls two fans, and adjusts their speed to maintain the enclosure temperature at 26-28 °C (**Fig. 1B**), a level close to adult mouse thermoneutrality of 30°C (Król & Speakman, 2003; Gaskill et al., 2012). Data were saved to local servers and streamed remotely for machine learning-based quantitative behavioral analysis along with annotations by several independent experienced human raters.

We monitored a total of 38 single-housed female mice continuously over 3-4 months, from first mating and first litter through subsequent breeding and litters (**Fig. 1C**, top). Although many different maternal behaviors were documented and scored over the postnatal period, we focused on interactions with pups and the nest in the first few days of each litter (**Fig. 1C**, bottom). This was due largely to surprising high pup mortality even in the protected conditions of the lab vivarium. We quantified litter survival from the moment of birth over the first 24 postnatal hours, in which most mortality was observed. For the first cohort of 15 mice, the average survival of the first litter was 62.2±12.1%. However, litter survival was clearly bimodal—7/15 first-time dams had 100% pup survival, and 5/15 dams had 0% pup survival (**Fig. 1D**, Litter 1). We refer to dams with >50% litter survival as 'high-pup-survival' dams, and dams with <50% pups surviving as 'low-pup-survival' dams.

87 We hypothesized that for low-pup-survival dams, litter survival rates might improve over
88 subsequent litters as dams learned from experience or had other forms of maternal adaptation in
89 the service of improved caregiving. However, this was not the case. As shown in **Figure 1D**, litter
90 survival of singly-housed C57Bl/6 mice continued to be bimodal, with 9/15 high-pup-survival
91 dams and 6/15 low-pup-survival dams for litter 2, 5/15 low-pup-survival dams for litter 3, and 4/8
92 low-pup-survival dams for litter 4 (**Fig. 1D**, left). With the exception of a single animal, high-pup-
93 survival dams continued to have high litter survival rates across all 3-4 monitored litters, and low-
94 pup-survival dams consistently lost most or all of their litters across all monitored litters (**Fig. 1D**,
95 right). This meant that the litter survival rate of the first litter predicted with high accuracy the
96 survival of subsequent litters (**Fig. 1E**; N=15, $r \geq 0.8$, $p<0.001$).
97
98 Inspection of video recordings showed that almost none of the litter mortality observed in either
99 high- or low-pup-survival dams was due to aggression or mauling of otherwise healthy pups
100 (observed in 3/38 dams). Instead, low-pup-survival dams neglected pups and nests after initial
101 nest-building was complete. In contrast, high-pup-survival dams consistently re-engaged in nest-
102 building, especially because entering and leaving the nest sometimes disrupted nest integrity. We
103 found that hand-scoring nest quality 1-3 days prior to parturition also predicted litter survival
104 across dams (**Fig. 2A**).
105
106 The importance of nest quality likely reflects the function of nests to keep pups insulated especially
107 when the dam is not present in the nest, because pups do not reliably thermoregulate their body
108 temperature and can perish from hypothermia (Weber and Olsson, 2008). However, it proved
109 challenging to reliably hand-score nests over extended periods, because nest structure was dynamic

and the visual features of a nest might not perfectly relate to the thermal insulative qualities that nests must provide. Therefore, we used thermal imaging to monitor pup temperature directly. This required us to segment the nest from the video recording and use it to isolate the temperature of the pups within the nest (**Fig. 2B**).

We monitored pup temperature during periods when dams made excursions from the nest. This happened frequently as mother animals left to forage for food and water, and to regulate their own body temperature as they can overheat if they stay too long on the nest (Leon et al., 1978; Gamo et al., 2016; Król and Speakman, 2019). We focused on moderate-duration excursions of >23 seconds, averaging pup temperature and heat loss over this period. We found that both the initial temperature and the temperature drop predicted pup mortality: higher tonic temperatures and less average heat loss were correlated with higher pup survival (**Fig. 2C,D**).

On average the total time spent on the nest was unrelated to litter survival rates (**Fig. 2E**). Initial time spent nest-building also was uncorrelated with litter survival (**Fig. 2F**). Instead, the main post-partum predictor of pup survival was amount of time the dam spent rebuilding the nest (**Fig. 2G**). Low-pup-survival dams typically spent some time nest-building initially, but then gave up, and subsequently lost most or all of their offspring within hours. High-pup-survival dams continued to adjust and rebuild the nest especially after leaving the nest and disrupting nest integrity. We also noticed that many high-pup-survival dams stocked food pellets near the nest (**Fig. 2H**). This seemed less related to shorter foraging times and more related to structural bolstering of nest wall integrity due to the weight of the food pellets.

133 It was surprising that over multiple rounds of breeding, low-pup-survival dams did not increase
134 attention to pups and time spent rebuilding their nests. We wondered if low-pup-survival dams
135 somehow could not provide adequate caregiving under any circumstance, or if instead perhaps
136 with different experiences these animals could become less neglectful and have higher survival
137 rates. Previously we documented the emergence of alloparenting in initially-neglectful and pup
138 averse virgin female C57Bl/6 mice, after being co-housed with experienced dams and litters
139 (Marlin et al. 2015; Carcea et al. 2021). In this case for alloparenting, behavioral changes in the
140 virgin were due to encouragement or coercion by the experienced dam, who shepherded or forced
141 the virgin to spend time in the nest until eventually the virgin became a successful alloparent.
142
143 We reasoned that similar parental behavior might emerge if we co-housed an initially low-pup-
144 survival dam with an experienced dam and her litter. We identified a separate cohort of low-pup-
145 survival dams with 0% litter survival at litter 1 and <50% litter survival for litter 2. Prior to mating
146 these animals for litter 3, we co-housed some of these animals for 16-20 days with an experienced
147 high-pup-survival dam and litter; other animals were kept isolated for a similar duration. We then
148 bred these animals and monitored pup survival for litters 3 and 4 when they were returned to being
149 singly-housed in our behavioral monitoring chambers (**Fig. 3A**).
150
151 We found that temporarily co-housing 'low-pup-survival' dams with another experienced mother
152 led to much higher survival rates of litters 3 and 4, compared to the other animals who were just
153 kept isolated for 16-20 days (**Fig. 3B**; red, co-housed dams, litter 3 survival: 88.5±4.8%, N=9;
154 black, isolate dams, litter 3 survival: 2.5±5.6%, N=5, $p<10^{-4}$ compared to litter 3 survival of co-
155 housed animals, Student's unpaired two-tailed t-test). Inspection of video recorded during the co-

housing period revealed that while the low-pup-survival dams were initially pup-averse, within hours to days they began interacting with pups and helped with childcare. This seemed to be due to behaviors of the other experienced dam, who shepherded the low-pup-survival dam into the nest and seemingly demonstrated aspects of maternal care (Carcea et al. 2021). This improvement of maternal care in the low-pup-survival dams did not seem to be due to, e.g., social buffering effects from co-housing with any other animal. Co-housing the low-pup-survival dam between litters 2 and 3 with either a pup-naïve virgin female or an experienced paternal male did improve pup survival for litter 3 (**Fig. 3C**); instead, there was a significant reduction in litter survival after co-housing with a naïve virgin female. Co-housing in these cases occurred without pups, as none of the adult animals would be lactating.

Our study provides insights into mouse maternal behavior over time and across consecutive litters, the impact of the nest and its insulating capacity on pup survival, and the role of socially-transmitted parental behavior via co-housing with an experienced mother animal. These observations resulted from the 'documentary' approach to monitoring animal behavior continuously over extended periods of days to months, with multiple cameras including under the nest. This was essential to accurately capture moments of parturition, to identify the number of pups delivered by each dam and the caregiving behaviors or neglect that resulted.

Our findings indicate that even under the controlled and protected conditions of a laboratory vivarium, childcare can be immensely challenging and taxing, requiring continued effort on the part of caregivers to ensure pups are fed and warm. Under the conditions of our environmentally-controlled home cages, approximately a third to a half of C57Bl/6 mouse mothers were observed

179 to lose most to all of their litters due to neglect of pups and nest. The nest structure was quite
180 dynamic, needing to be repeatedly adjusted or rebuilt due to the movement of the mother in and
181 out of the nest, and the motion of the pups. Thermal imaging was important to relate this aspect of
182 parental behavior to litter survival, as pups rapidly dropped in temperature without a well-
183 constructed insulating nest and died within minutes.
184
185 One potential concern is that the extent of pup mortality and relatively high prevalence of low-
186 pup-survival dams is a consequence of sustained single-adult housing especially combined with
187 the stress of childcare. A related caveat is that our studies were performed exclusively in C57Bl/6
188 mice, and it is known that there are differences in parental behaviors across rodent strains (Ward,
189 1980). Even accounting for the genetic background, within the C57Bl/6 strain there was
190 considerable bi-modality in childcare abilities, which might reflect other individual differences in
191 gene expression and/or neural responses to infant distress cues that will need to be further
192 documented. Most wild species of mice are believed to live in multi-female colonies (Ferrari et
193 al., 2019) where perhaps many different adults can participate in childcare, tending to pups and
194 nests, and shepherd or help other inexperienced adults take care of offspring. However, little is
195 known about the fine-scale adult social dynamics of colony organization and how this relates to
196 communal child-care. The system we have developed enables a systematic and parametric
197 exploration of different variables related to social structure and housing conditions, and promises
198 to reveal much more about the family life of lab mice and other species.

References and Notes

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Author contributions: L.S. and A.M. built the 24/7 monitoring system. L.S. performed behavioral experiments. L.S., R.H., S.S., D.A., M.K., and P.L. performed behavioral analysis. K.E.A. provided analysis tools and data archiving. L.S., R.C.F., K.E.A., and A.M. designed the study and wrote the paper.

251 **Materials and Methods**

252 All procedures were approved under NYU Grossman School of Medicine IACUC protocols.

253

254 **System for Long-Term 24/7 Behavioral Analysis.** For long-term monitoring of the acquisition
255 and expression of maternal behavior in single-housed animals, we used 8–10-weeks-old C57BL/6
256 naïve virgin female mice. No statistical methods were used to predetermine sample size. The
257 experiments were not randomized. The behavioral raters were blinded to litter survival rate,
258 allocation during co-housing, and outcome assessment.

259 A virgin female mouse was housed alone in a 27.94 × 27.94 × 27.94 cm plexiglass
260 homecage covered with plentiful bedding material, food pellets, hydrogel and/or a water bottle
261 (refreshed every 3-5 days), and one Nestlet. We began by recording continuous video of the naïve
262 virgin behavior while housed alone for up to 5 days, before introducing an experienced C57BL/6
263 male mouse for breeding afterwards. The male mouse was removed from the enclosure after
264 successful intercourse, confirmed through visual examination of video footage, typically after 24-
265 48 hours. For further confirmation of mating, when removing the male mouse from the enclosure,
266 we also checked the virgin for a vaginal plug and we closely monitored the female mouse for
267 physical and behavioral changes typical of a gravid animal. If by day 10 post-mating, the female
268 mouse did not show weight-gain or weight-redistribution, a new experienced male was introduced
269 in the homecage for mating.

270 Dams were allowed to stay with the pups until weaning on P21 in the same homecage.
271 After that, the female was placed back in the enclosure for a few days and mated again, following
272 the same procedure as with the first litter. This was performed for four consecutive litters.

273

Video Acquisition. Video recordings were acquired from 1-3 cameras (OV5647 sensor) sensitive to infrared light and outfitted with 160 FOV wide-angle lenses and placed either above the enclosure (overhead view), perpendicular to the nest (nest view), or below the nest (bottom-up view). Each camera was connected to a Raspberry Pi 4 single board computer that captured, streamed, and saved uninterrupted video from the enclosures (30 frames per second) to a local server using the Python library OpenCV. For some animals, videography was performed continuously at all times up to weaning of the third litter; for other animals, we focused video recording on 3-5 days before birth up until P5.

Behavioral Analysis. For analysis of video data, we used two different DeepLabCut (DLC) networks: one trained to track the location of the dam and another one to track the pups. The location of the nest was extracted with custom Matlab code using image segmentation, and we processed frame-by-frame pose data obtained with DLC to identify episodes of dam nest entry or exit. Nest entry time was defined as the moment when the two front paws entered the nest cavity. We also used nests as a region of interest (ROI), to identify when a pup was out of the nest and how long it took for the dam to it.

We used Datavyu to manually score other behaviors. Five independent coders identified relevant individual and social behaviors and scored the videos blind to litter number and survival rate. The results from each rater were compared, compiled, and cross-validated.

Thermal Imaging. For thermal imaging, we recorded continuous thermograms with a thermal camera (FLIR C3) placed above the nest, streaming temperature data (15 frames per second) to a

Windows PC through a USB cable. Thermographic data were captured with the FLIR Tools software, timestamped, and saved as a csv file.

We synchronized the thermograms and the video recordings post-hoc using timestamp information. We isolated the episodes in which the dam was out of the nest, identified the video frame and thermogram corresponding to the onset of the episode, located the nest opening using image segmentation, and then extracted and averaged the corresponding pixel temperature information from that ROI. We saved these frame-by-frame average traces as csv files. For pup temperature analysis, we identified episodes in which pups were out of the nest and followed a similar procedure to the nest temperature analysis focusing on the out-of-nest pup as our ROI.

Litter Mortality. We monitored both the videos and the thermograms daily to establish how many pups were born alive, how many were stillbirths, and how many were cannibalized. We used color, temperature, movement, and physical integrity to determine if pups were alive. We calculated the litter survival rate as surviving pups on P5 divided by the total number of pups born alive.

Co-Housing. Dams with 0% litter survival during their first litter and less than 50% survival during their second litter were co-housed either with an experienced high-pup-survival dam with her pups (~P5 and older), a virgin, a paternal male, or left in isolation. Co-housing was performed for 16-20 days, before being mated a third and fourth time.

Data and Code Availability. The data that support the findings of this study are available on Databrary. Custom code are available on Github.

Figures and Figure Legends

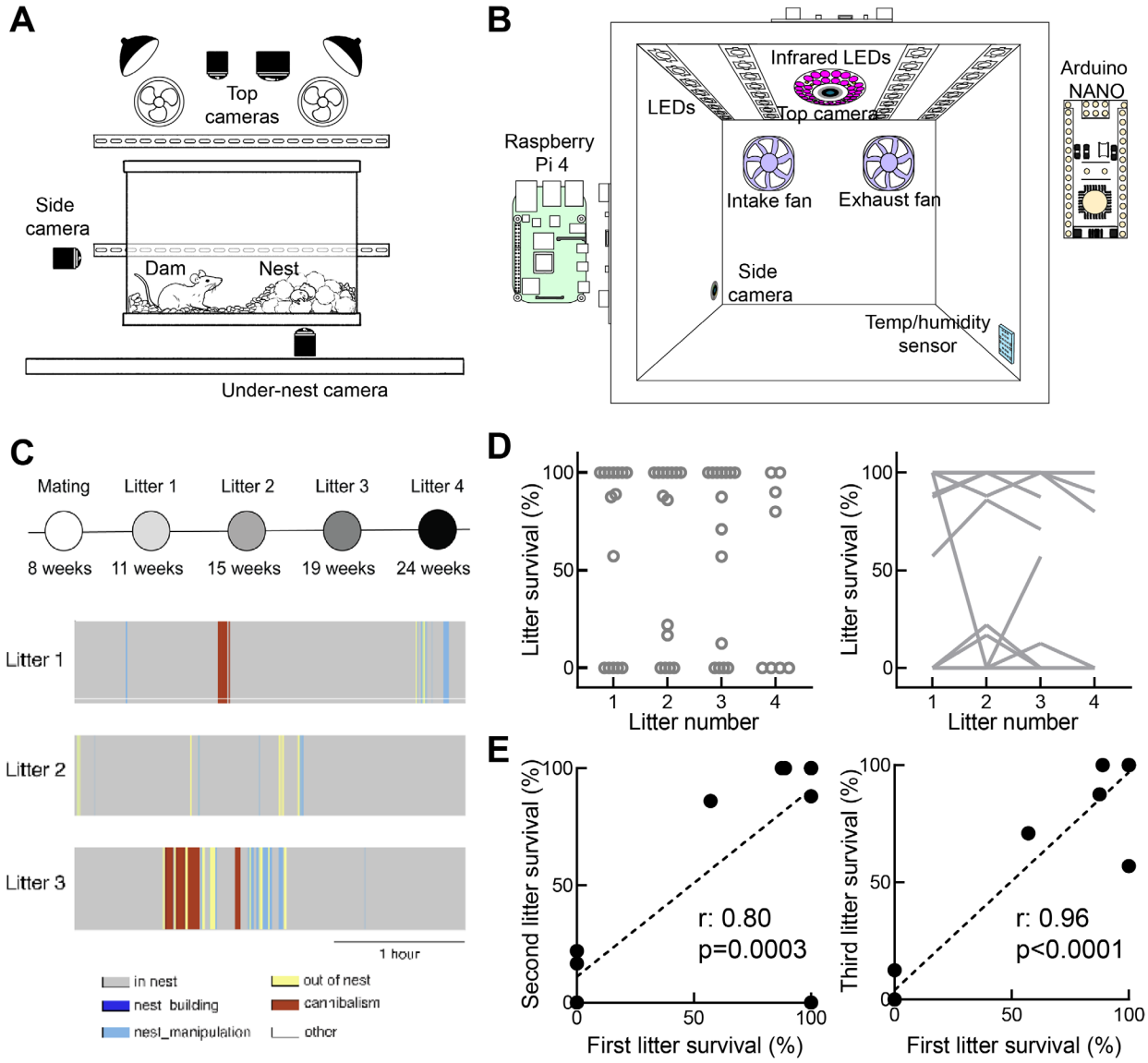

Figure 1. Continuous long-term monitoring of mouse maternal care across litters. **A**. Schematic of setup for 24/7 continuous monitoring of homecage behaviors. A standard mouse homecage was placed within a custom enclosure containing cameras from top, side, and beneath the nest location. **B**. System for long-term continuous imaging and environmental controls. **C**. Homecage behaviors were monitored over four litters, scored by automated methods (in/out of nest) and manually (nest building, nest manipulation, cannibalism). Top, timeline of monitoring of individual animals

(times are ages of the dams). Bottom, example ethograms showing several hours of spontaneous homecage behaviors for the same animal during litters 1, 2, and 3. **D**. Litter survival at 24 hours post-parturition. Left, fraction of pups surviving after the first postnatal day (N=15 dams). Each gray circle represents one dam's litter, with the same animals monitored over litters 1-4. 7/15 dams had 100% survival at litter 1 ('high-pup-survival dams'), and 6/7 of those animals had 100% survival at litter 3 as well. 5/15 dams had 0% litter survival at litter 1, and 4/5 of those animals had 0% survival at litter 3. Right, same data with lines connecting litter survival from the same dam across all 3-4 monitored litters. **E**. Correlation of litter survival over litters. Left, litter 1 survival predicted litter 2 survival across dams (N=15, r(DF)=.80, p=.0003). Right, litter 1 survival also predicted litter 3 survival (N=15, r(DF)=.96, p<0.0001).

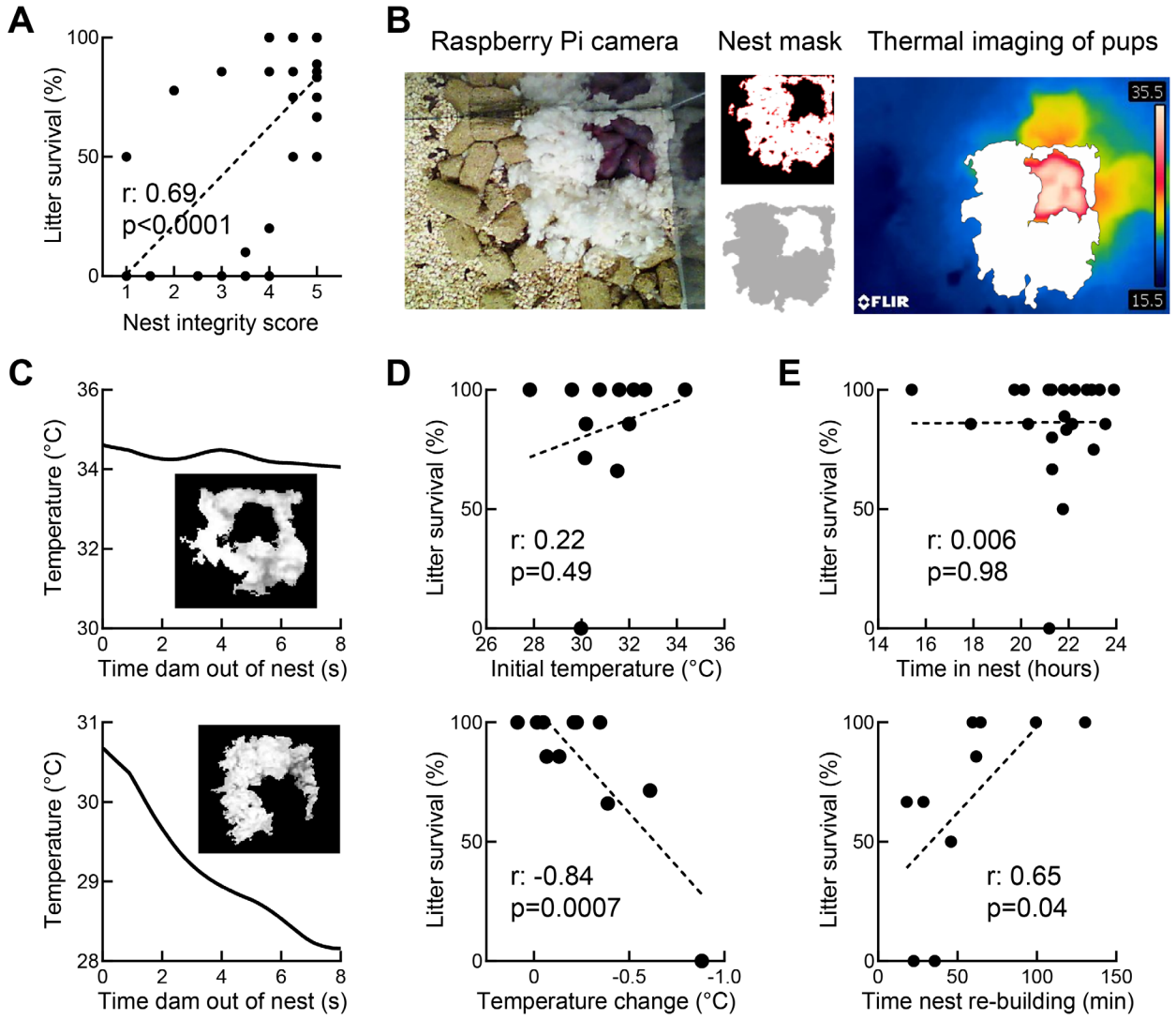

Figure 2. Nests must be rebuilt to ensure pup thermoregulation. **A**. Visual scoring of nest quality; higher nest integrity scores were correlated with higher litter survival rates (r(DF)=.69, p<.001). **B**. Procedure for monitoring pup temperature. Video frames were used to compute nest mask, registered with thermal imaging to isolate pup temperatures. **C**. Examples of heat loss when dam leaves the nest; curves show average drop in pup temperature over many excursions of the dam from the nest (8+ seconds in duration). Top, high-pup-survival dam; bottom, low-pup-survival dam. Insets show representative nest structures from these animals. **D**. Pup temperature when dam left the nest. Top: litter survival was not correlated with the initial temperature upon dam leaving,

*r(*DF)=.22, *p*=0.49. Bottom: litter survival was correlated with temperature drop after dam left the nest, *r*(DF)=-.84, *p*=.007). **E**. Dam interaction with the nest. Top: litter survival was not correlated with total time spent in the nest on the first full day after parturition, *r*(DF)=.006, *p*=.98. Bottom: litter survival was correlated with amount of time dams spent rebuilding the nest, *r*(DF)=.65, *p*=.04.

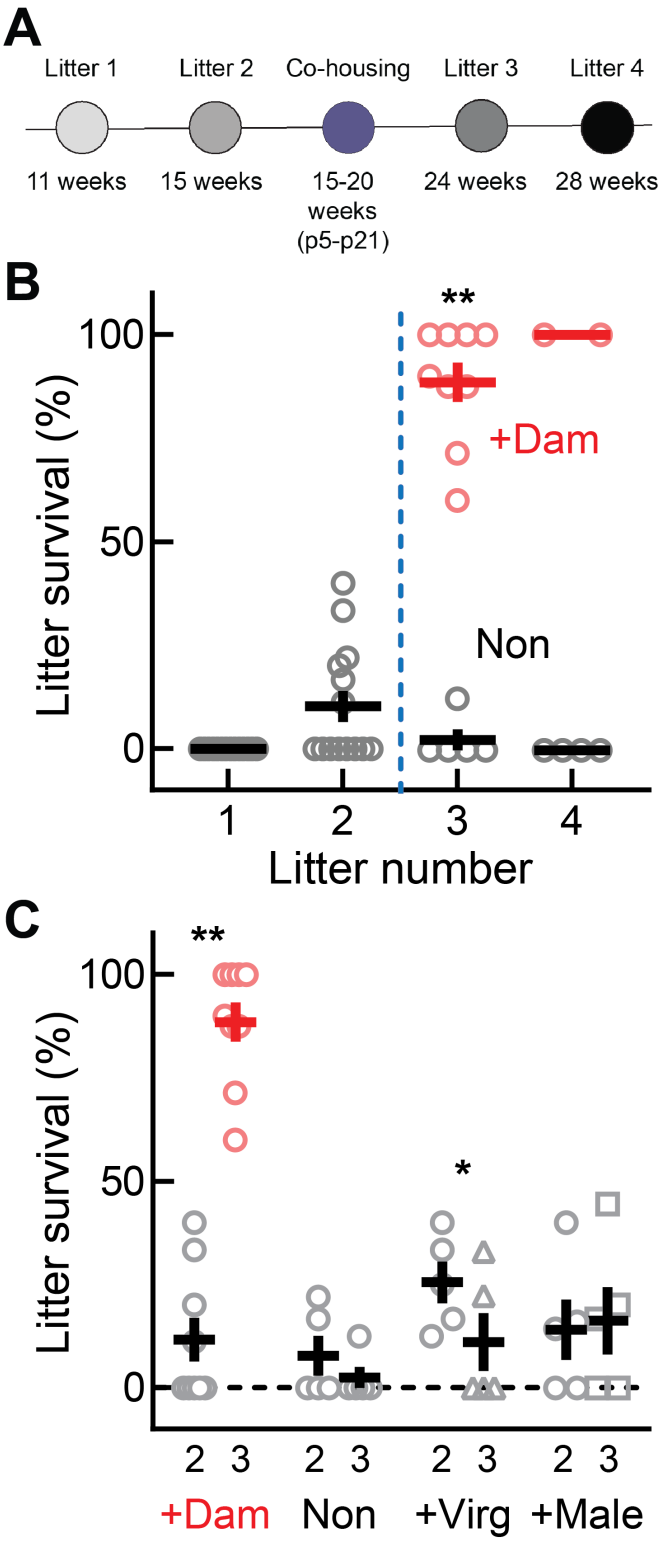

Figure 3. Co-housing with an experienced high-pup-survival dam improves maternal care. **A**, Timeline for co-housing studies: virgin females were bred and those dams with 0% litter survival

353 for litter 1 and <50% survival for litter 2 were selected for these studies; dams were singly-housed
354 for all four litters. Between litters 2 and 3, dams were divided into four groups and placed in a
355 separate cage for 16-20 days before the third round of mating ('+Dam': housed with an
356 experienced high-pup-survival dam and her litter; 'Non': non-co-housed, i.e., singly housed
357 without pups; '+Virg': co-housed with a pup-naïve virgin female but no pups; '+Male': co-housed
358 with an experienced paternal male but no pups). **B**, Co-housing with an experienced high-pup-
359 survival dam led to improved litter survival of initially low-pup-survival dams. Litter survival of
360 all animals for litter 1: 0%, for litter 2: 10.2±3.7% (N=14). Nine of these animals were co-housed
361 prior to mating for litter 3; litter survival for litter 3: 88.5±4.8% (N=9), for litter 4: 100% (N=2).
362 For the five non-co-housed animals from the same cohort, litter survival remained low; for litter
363 3: 2.5±5.6% (N=5, $p<10^{-4}$ compared to litter 3 survival of co-housed animals, Student's unpaired
364 two-tailed t-test), for litter 4: 0% (N=4). Litter 2 survival for these two groups was not significantly
365 different (dams to be co-housed, litter 2 survival: 11.6±5.3%; non-co-housed dams, litter 2
366 survival: 7.7±4.8%; p=0.60, Student's unpaired two-tailed t-test). **C**, Comparison of litter survival
367 for litters 2 and 3 per group ('+Dam', litter 2 survival: 11.6±5.3%, litter 3 survival: 88.5±4.8%,
368 N=9, $p<10^{-4}$ significant increase, Student's paired two-tailed t-test; 'Non', litter 2 survival:
369 7.7±4.8%, litter 3 survival: 2.5±5.6%, N=5, p=0.45; '+Virg', litter 2 survival: 25.5±5.1%, litter 3
370 survival: 11.0±7.0%, N=5, p=0.02 significant decrease; '+Male', litter 2 survival: 14.1±7.3%, litter
371 3 survival: 16.2±8.2%, N=5, p=0.80). **, p<0.01; *, p<0.05.